Improvement of the yeast slide culture technique

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RESUMO

Este trabalho tem como objetivo melhorar a técnica de cultura em lâmina para ser usada na avaliação da viabilidade de leveduras sob diferentes condições fisiológicas.

Inicialmente, foram otimizadas as condições ideais para o cultivo em lâmina de uma estirpe laboratorial (BY4741) e de uma estirpe industrial (NCYC 1214) da levedura *Saccharomyces cerevisiae*. O melhor protocolo foi obtido utilizando: YEPD agar com uma espessura de cerca de 2 mm; 20 µL de uma suspensão de 1 x 10^5 células/mL para a estirpe BY4741 ou de 5 x 10^4 células/mL para a estirpe NCYC 1214; uma câmara de humedecimento com 100 µL de água desionizada e um tempo de incubação de 24 h, a 25 ° C.

Com o objetivo de facilitar a contagem das microcolónias, foi adicionado um corante (*calcofluor white*, CFW) ao meio YEPD agar. Ensaios preliminares, em YEPD líquido, contendo diferentes concentrações de CFW, permitiram verificar que o corante, até 5,0 µg/L, não inibe o crescimento da levedura. Uma concentração de 2,5 µg/L de CFW permitiu a coloração da parede das leveduras, não se observando células com morfologia alterada, sendo esta a concentração de CFW selecionado nos estudos subsequentes.

A técnica de cultura em lâmina, com ou sem CFW, foi aplicada para avaliar a viabilidade de células saudáveis (células em fase exponencial de crescimento), células submetidas a *stress* de etanol [células expostas a 20% (v/v) de etanol, a 25 ºC, durante 2 h] e células envelhecidas (células incubadas em água, a 25 ° C, durante 48 h), da estirpe laboratorial. A percentagem de células viáveis não foi significativamente diferente entre as duas técnicas (com ou sem CFW), após uma incubação de 24 horas. Finalmente, a técnica de cultura de lâmina, contendo CFW, foi comparada com duas técnicas habitualmente usadas na indústria cervejeira: fermentação de curta duração e determinação da percentagem de células gemuladas. Os resultados obtidos através da técnica de cultura de lâmina, desenvolvida, seguem um padrão similar aos obtidos nos ensaios de fermentação de curta duração e aos da determinação da percentagem de células gemuladas.

Os resultados obtidos sugerem que a técnica de cultura em lâmina, combinada com CFW, parece ser uma alternativa, fácil, rápida (em 24 h) e reprodutível, relativamente ao método convencional (técnica de plaqueamento), para a avaliação da viabilidade de células de levedura. Deverá ser realizado trabalho adicional a fim de validar o método com estirpes industriais.

Palavras-chave: *calcofluor white*; contagem de microcolónias; *Saccharomyces cerevisiae*; viabilidade; levedura

ABSTRACT

This work aims to improve the slide culture technique for applying on the evaluation of yeast viability under different physiological conditions.

Firstly, the optimal conditions for slide culture were standardized for a laboratorial (BY4741) and an industrial strain (NCYC 1214) of the yeast *Saccharomyces cerevisiae*. The best protocol was achieved applying: YEPD agar with a layer thickness of approximately 2 mm; 20 µL of a suspension of 1 x 10^5 cells/mL for the strain BY4741 or 5 x 10^4 cells/mL for the strain NCYC 1214; a wetting chamber with 100 µl of deionized water; and an incubation time of 24 h, at 25 °C.

With the objective to facilitate the micro colonies counting, a dye (calcofluor white, CFW) was added to YEPD agar. Preliminary experiences in YEPD broth containing different CFW concentrations allowed to verify that the dye did not inhibited the grow up to 5.0 µg/L. As 2.5 µg/L CFW allowed the staining of the yeast cell wall, and no cells with aberrant morphology were observed, it was the CFW concentration selected for further studies.

The slide culture technique developed, with or without CFW, was applied to evaluate the viability of healthy cells (cells in exponential phase of growth), ethanol stressed cells [cells exposed to 20 % (v/v) ethanol, at 25 ºC, for 2 h] and aged cells (cells incubated in water, at 25 ºC, for 48 h) of the laboratory strain. The percentage of viability did not differ significantly for both techniques (with or without CFW) tested after 24 h of incubation. Finally slide culture combined with CFW was validated by comparing with the short fermentation and budding assays, two common techniques used in brewing industries. The percentage of viability obtained with the developed technique was in accordance to the short fermentation assay and in some extent to the budding index.

The results obtained suggest that the slide culture technique combined with CFW seems to be an easy, fast (within 24h), reproducible and reliable alternative to conventional method (standard plate count technique) for the assessment of viability of yeast cells. More work should be carried out in order to validate the method with industrial strains.

Keywords: calcofluor white; micro colony count; *Saccharomyces cerevisiae*; viability; yeast

WORK OBJECTIVES

This work aims to improve the slide culture technique in order to obtain an easy, fast, reliable and reproducible assessment of yeast viability. Thus, several parameters will be standardized: YEPD agar recipe, thickness of YEPD agar layer, cell concentration, incubation time, humidity of wetting chamber and the criterion for counting the microcolonies. Additionally, for facilitating the microscopic visualization of the microcolonies a dye will be added to YEPD agar. Finally, the improved slide technique will be applied to a laboratory yeast strain of *Saccharomyces cerevisiae* in different physiological conditions and compared with conventional techniques like short fermentation test and budding index, commonly used in the brewing industry.

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1. Introduction

The evaluation of yeast viability is of utmost importance in fermentation industries. The slide culture is one of the many techniques used to assess yeast viability. This technique is very useful because the assay is more reliable than staining methods and less time consuming than the standard plate count method (Lentini 1993). An overview of yeast viability and vitality methods is given and the importance of these parameters in the fermentation industry is stressed. Also general properties of the yeast *Saccharomyces cerevisiae* are summed up for the understanding of the mechanisms of certain viability and vitality methods.

1.1 Cell structure and behavior of *Saccharomyces cerevisiae*

S. cerevisiae is one of the most employed species in fermentation industries, namely in brewing and ethanol production. It is also widely used as eukaryotic model organism. Yeast cells multiply by a process called budding. Additionally, some yeast strains also tend to flocculate and form big flocs.

1.1.1 Cell wall composition and flocculation behavior of *Saccharomyces cerevisiae*

Certain yeast strains have flocculent behavior. Flocculation is defined as the aggregation of yeast cells into multicellular masses, called flocs. The process is nonsexual, homotypic (only one type of cells is involved) and reversible (Soares 2011). The flocculation behavior is dependent on the yeast cell wall composition.

The yeast cell wall is roughly composed of an inner and an outer layer. The inner layer consists mostly of β-glucan and chitin and the outer layer of α-mannan (Soares 2011). At physiological pH, due to the presence of ionized carboxyl's of the cell wall proteins and phosphodiester groups of the phosphomannans , the net charge of the cell walls is negative which keep apart cells from each other (Soares 2011). Thus flocculants have special cell wall properties in order to facilitate flocculation.

In flocculent yeast, lectin-like proteins are present in the cell walls. These lectin-like proteins interact with the carbohydrate residues of the α-mannans in presence of Ca^{2+}-ions (Miki et al. 1982). Flocculation is very useful in some industrial processes. When the cells flocculate and settle, the yeast can easily be separated (Soares 2011). But in laboratory circumstances flocculation is sometimes unwanted. In that case, a complexing agent like EDTA can be added to complex with Ca^{2+} -ions and suppress the flocculation.

1.1.2 Budding process of *Saccharomyces cerevisiae*

Budding is the asexual reproduction of yeast cells. The bud first emerges from the mother cell and then cells separate. The mother cell exhibits a bud scar and the daughter cell a birth scar. The asexual reproduction process is important in order to maintain a single variety of yeast strain.

When budding, a chitin ring is firstly formed on the place of the emerging bud. Later, the primary septum, the secondary septum and the lateral wall chitin are formed, respectively (Figure 1) (Orlean 2012).

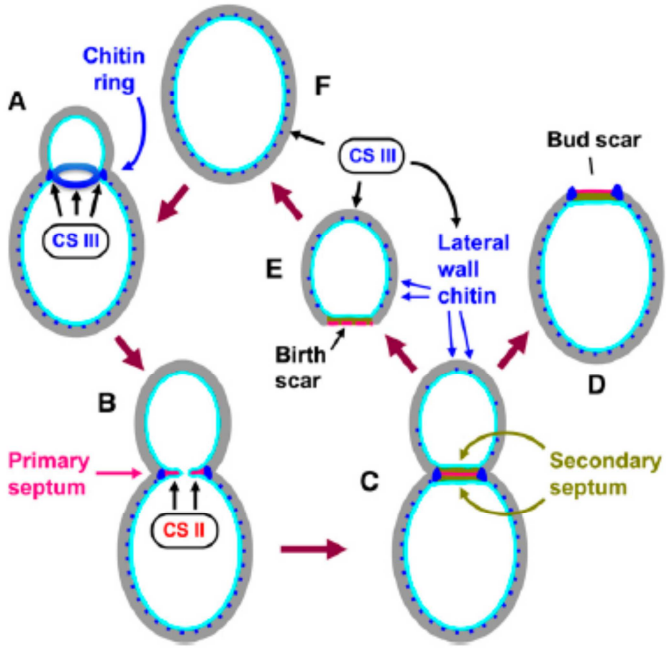

Figure 1: Chitin synthase II and III play a role during the budding process of *S. cerevisiae*. (A) A chitin ring is formed (CS III). (B) The primary septum is synthetized (CS II). There should be noted that no chitin is synthetized in the lateral cell walls of the bud yet. (C) Secondary septum is formed and CS III provokes the synthesis of chitin in the lateral cell wall of the bud. (D) The cell separates and most of the chitin material sticks to the mother cell. (E and F) Lateral wall chitin is formed (CS III) while the daughter cell is growing. Source: Orlean (2012).

1.1.3 The yeast *Saccharomyces cerevisiae* as a cell model

In research model organisms are often used to optimize methods and develop standard practices. Karathia et al. (2011) compared *S. cerevisiae* to 704 other species and concluded that they could suit as a model organism. Even animals and *Homo sapiens* in particular have a significant fraction of common biological processes (Karathia et al. 2011). For example, 30 % of the human disease genes have orthologues in *S. cerevisiae*. The main advantages of using *S. cerevisiae* are: 1) ethical constraints are avoided by using yeast as model organism; 2) *S. cerevisiae* is easy to grow in laboratory circumstances; 3) the cell model and the genome of *S. cerevisiae* are well known and 4) *S. cerevisiae* has many orthologues in other organisms (Karathia et al. 2011).

1.2 Definition of viability and vitality

Viability and vitality are defined in a number of ways. In order to strictly outline the concepts of viability and vitality, first the meaning of life will be discussed. Keeping in mind there is no clear-cut answer to this; arbitrary rules will be set to establish unambiguous definitions of viability and vitality.

What does life mean? It is not a straightforward question, especially when talking about smaller organisms. A microorganism can either be dead, alive or even somewhere in between (Figure 2). A living cell undergoes many degradation steps before being dead. At the opposite a damaged cell is able, only until the degradation has reached a certain point, to recover itself when introduced in a more favorable environment (Lodolo and Cantrell 2007). As the exact point of death and the point of no return is unknown, it is hard to define either a cell is dead or alive (Davey 2011).

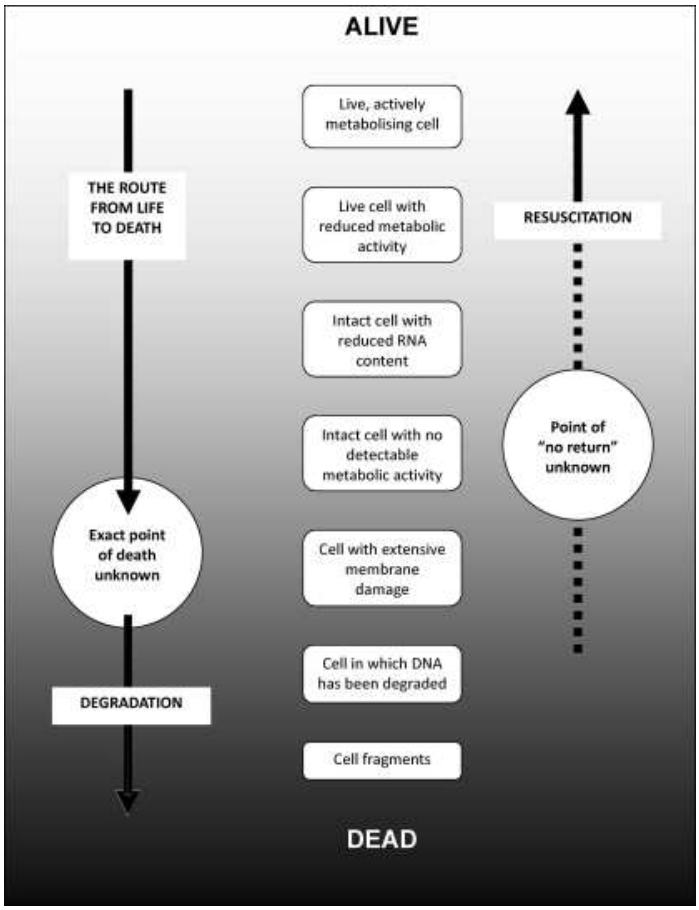

Figure 2: A micro-organism can either be dead, alive or somewhere in between. Source: Davey (2011).

A first definition of viability is the ability of the cell to reproduce. The capability of cells to multiply is routinely tested with culture techniques (Davey 2011). The consecutive budding of the yeast cell results in a (micro) colony and therefore indicates its viability. Secondly, viability can be defined as the conservation of metabolic activity. Dyes such as methylene blue and fluorescein-diacetate indicate metabolic activity when conversion takes place by intracellular enzymes (Heggart et al. 2000). In third place a cell can also be defined viable when the cell membranes remain intact. Trypan blue is a dye able to penetrate and color the cell when membranes are damaged but is excluded when membranes are intact (Heggart et al. 2000). Life or death cannot clearly be defined. Although three possible definitions of viability are given: 1) the ability to reproduce; 2) the conservation of metabolic activity and 3) the integrity of the cell membranes.

When stained, yeast cells sometimes are not homogenously colored as result of a difference in vitality of the viable cells. Vitality is a measure for the activity of a living cell (Heggart et al. 2000). The vitality of cell is strongly dependent on the environmental conditions of the cell. Thus, when assessing vitality it is important to measure in standardized conditions (Heggart et al. 2000). Regarding that, three definitions of vitality are proposed: 1) the activity of viable cells under certain physiological conditions; 2) the ability to still perform under stressed conditions and; 3) the capacity to reinitiate activity when transferred from a poor nutrient to a rich environment (Lodolo and Cantrell 2007).

1.3 Importance of viability and vitality assessment in fermentation industry

Regarding industrial fermentation processes the viability and vitality of the yeast is of great relevance, especially when yeast is recovered and reused in next fermentations. Viability and vitality assessments and its practical uses in the brewery and in the biofuel industry are discussed as well as yeast handling in breweries.

1.3.1 Yeast handling in breweries

During fermentation of pitched wort the yeast is exposed to different types of stress. Due the rising of ethanol and CO_2 concentration and the decrease in nutrients the yeast tends to give up and flocculates. Ideally the yeast must have a pre-defined flocculation behavior. After flocculation it is possible to recover the yeast for a certain number of generations (Figure 3).

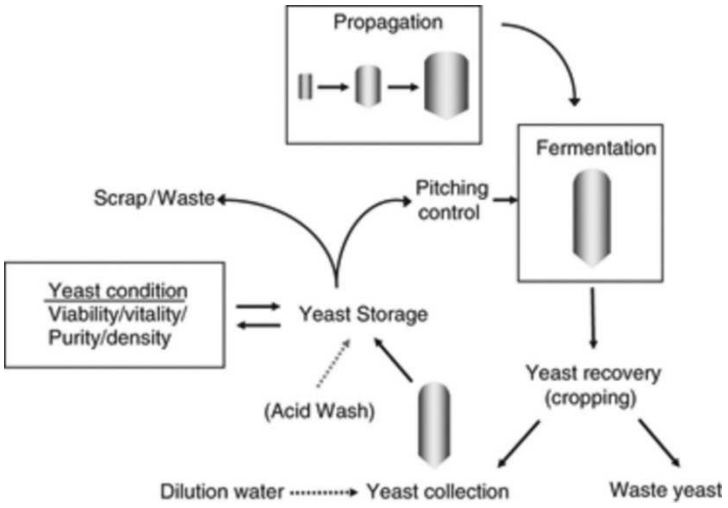

Figure 3: The main activities during yeast handling. Source: Lodolo et al. (2008).

Usually, after fermentation a yeast slurry of three layers is obtained. In case of bottom fermenting yeast, the bottom layer consists of dead yeast cells and other trub particles such as polyphenol complexes and hop particles, the middle layer of healthy yeast cells and the top layer of collapsed foam particles and yeast that settled at a later stage. The objective is only recovering healthy cells which can be achieved by decanting the unhealthy yeast. Because it is difficult to isolate the healthy cells and the crop can be contaminated further washing will be needed (Lodolo et al. 2008).

The yeast cells can be washed with sterile water, but more effective is acid washing. A typical acid washing is done below 4°C lowering the pH to 2.2 -2.5 with phosphoric acid (Lodolo et al. 2008). But acid wash can damage the yeast while the bacteria's are not killed (Lodolo et al. 2008). Also during storage the yeast quality can be affected. For good practice the yeast should be stored at 4 °C (Lodolo et al. 2008).

Cropping, washing and storage are three critical points during yeast handling in a brewery where viability or vitality assesments are of good use. Also in the bio-ethanol fuel industry yeast is often recycled.

1.3.2 Bio-ethanol fuel

S. cerevisiae is also used to produce bio-ethanol fuel. Mostly agriculture waste streams are used to produce bio-ethanol (Sanchez-Gonzalez et al. 2009). Under high ethanol concentrations the yeast is under stress and is unable to assimilate essential vitamins such as biotin (Ben Chaabane et al. 2006). To overcome that problem a two-phase bioreactor system can be used (Figure 4). The first reactor focuses on the growth of the yeast and the second reactor on ethanol production. In order to maintain high viability the yeast from the second reactor can be recycled by pumping back to the first reactor. An accurate and rapid viability assessment is also here of good use (Ben Chaabane et al. 2006).

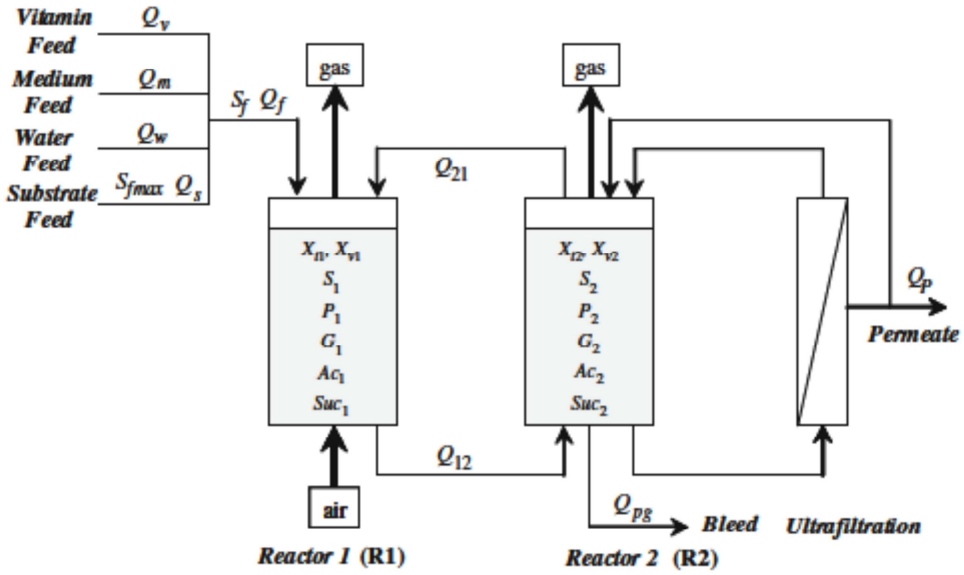

Figure 4: Example of two-phase bio-ethanol reactor. Source: Ben Chaabane et al. (2006).

1.4 Techniques for the assessment of viability and vitality

In the previous section the importance of an accurate and easy-to-use viability and vitality assessment was stated. Methods based on cell replication and techniques involving staining of cells are most commonly used. The accuracy of these techniques can be increased using automated counting systems such as image based equipment or flow cytometers (Davey and Hexley 2010). Applying these automated methods, operator related errors are strongly reduced. However a combination of different techniques tends to provide better results (Heggart et al. 2000). An overview of the most common techniques used to assess viability and vitality is given in Table 1.

At present no vitality assessment is available to test the overall physiological state of the yeast. Although methods based on metabolic activity, measurement of cellular components and fermentative capacity are helpful to assess vitality (Lentini 1993). The short fermentation test is performed doing fermentation on small scale and monitoring the cell density in time. The acidification power test is based on the ability of the yeast to acidify a glucose medium (Gabriel et al. 2008). In the next paragraphs the methods based on staining and cell replication will be treated in more detail.

Table 1: Techniques to assess viability and vitality. Adapted from Heggart et al. (2000).

Parameter	Methods based on	Examples
Viability	Cell replication	• Standard plate count • Slide count technique
	Staining	• Brightfield stains • Fluorescent stains
Vitality	Metabolic activity	• Vital staining • Acidification power test
	Measurement of cellular components	• ATP • NADH
	Fermentative capacity	• Short fermentation test

1.4.1 Culture techniques

As the ability of a cell to reproduce is a possible definition of yeast viability, cell replication methods are used. The biggest drawback of the cell replication techniques is the time needed for yeast growing. In order to establish a reproducible assessment, attention should be paid to the composition of the growing substrate. Changes in nutrient composition and surface properties such as the humidity of the growth medium can affect the yeast growth and therefore result in a reduced reproducibility of the viability assessment (Heggart et al. 2000). In some cases viable cells are even unable to replicate and provide false negative results. False negative results may also be caused by damaging of the cells, not using the right cell density or not giving the cells sufficient time to grow (Gilliland 1959; Davey 2011). Two different methods are distinguished: the standard plate count technique (SPC) and the slide count technique.

1.4.1.1 The standard plate count technique

The standard plate count technique involves the counting of colony-forming units (CFU's) on a nutrient agar plate after spreading a yeast suspension. A CFU is a cell that is able to give rise to a colony. It is easy to use but 2-4 days are required in order to obtain colonies that are visible by naked eye (Heggart et al. 2000). Considering that counting is operator defined, efforts are made to automate the counting. A possible way to automate the counting is the use of an image analyzer. That way the counting process is standardized, but the problem of time is not solved. The slide count technique makes the culture technique less time consuming.

1.4.1.2 The slide count technique

In order to grow yeast on a slide, a thin agar block can be used as substrate. By pipetting a cell suspension and covering with a microscope slide quantitative results can be obtained in less than one day (Heggart et al. 2000). A possible problem when using the slide count technique is false positive results. A colony can be observed by two or more cells coinciding at the same place but not being viable at all. Non-viable cells can clump together as it is the case of very flocculent yeast. For overcoming this problem, flocs of yeast are broken down by using EDTA (Heggart et al. 2000). A viable cell can also start the budding process but become non-viable before the budding process is completed. For that reason a non-viable cell gives the appearance to be viable (Orlean 2012). The slide count technique is reliable, but the technique should be well standardized in order to prevent possible problems (Lentini 1993).

1.4.2 Use of Dyes

A second group of viability assessments are the staining methods. The dyes can be classified in two groups: brightfield and fluorescent stains. The brightfield stain methylene blue (MB) and the fluorescent stain magnesium salt of 1-aniline-8-naphtalenesuphonic acid (Mg-ANS) are two stains recommended to use by the *American Society of Brewing Chemists* (Middlekauff et al. 1980; 1981). Dyes are in some cases also used to assess the vitality of the yeast, for example when the shade of the coloring is dependent on the activity of the yeast cell.

1.4.2.1 Bright field stains

When yeast cells are stained with a brightfield dye, the coloring can be dependent on the viability and vitality. The changes in color are observed with a light microscope. In table 2 four cheap brightfield staining methods are presented (Kucsera et al. 2000).

Table 2: Four cheap brightfield stains for determining yeast viability. Adapted from Kucsera et al. (2000).

Dye	Color viable cells	Color death cells
Methylene blue	Colorless	Blue
Trypan blue	Colorless	Blue
Phloxin B	Colorless	Red
Rose Bengal	Colorless	Light red

Methylene Blue

Methylene blue (MB) is a stain presenting blue color in the oxidized form. Viable cells are able to reduce MB to its colorless form when entering the cell (Figure 5). Therefore staining is linked to the reducing capacity of the yeast cell and the integrity of the cell membranes (Buchholz et al. 2008).

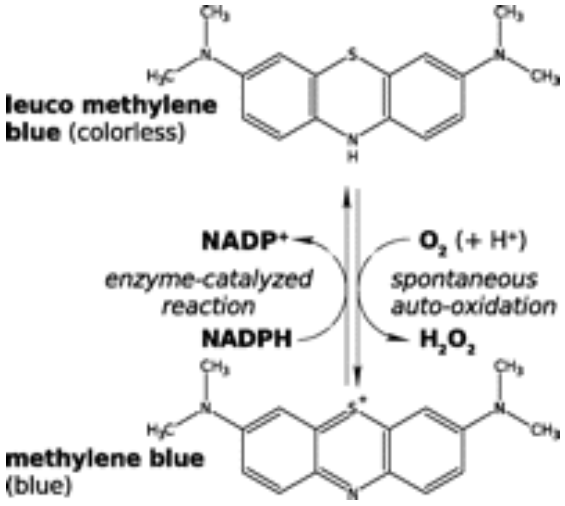

Figure 5: Oxidation/reduction of methylene blue. Source: Buchholz et al. (2008).

Viable cells are colorless because the enzyme-catalyzed oxidation of MB in the cytoplasm of the yeast cell is balanced with the influx rate of the dye. The influx rate of the dye in the cell varies between different strains of yeast or depend on its physiological condition (Jones 1987). A higher influx rate requires a higher metabolic activity in order to convert the dye in its colorless form. In non-viable cells no active reductases are present and the membranes are likely to be damaged. Therefore non-viable cells are colored blue (Smart et al. 1999).

Several methods are available to stain yeast cells with MB. The conventional aqueous MB method (0.01 % w/v) in distilled water is only accurate for viabilities above 90 % (Middlekauff et al. 1980; Heggart et al. 2000). When the viability is below 90 % the yeast viability is seriously underestimated. Therefore a number of variations of the MB method have been developed (Heggart et al. 2000). *ASBC* recommends using the phosphate buffered MB method as the accuracy is better with viabilities below 90 % (Middlekauff et al. 1980). Another drawback of the conventional aqueous method is that the cell is not homogenously colored, which leaves the observing open for interpretation. With the use of citrate methylene violet the coloring is more homogenously and the problem can be solved (Smart et al. 1999). Sometimes the different shades of coloring are useful in order to assess vitality. In that case MB can also be used in combination with *Safranin 0* in order to estimate vitality (Smart et al. 1999).

Trypan blue

Trypan blue (TB) is also used for the staining of non-viable cells. The dye exists in two tautomeric forms (Figure 6). In water, TB presents the keto-hydrazone form and has a blue color (Graham et al. 2013). The substance only colors cells with damaged membrane (dye exclusion method). As TB is negatively charged it is not able to permeate through an intact cell membrane (Tran et al. 2011). But TB is a relatively small molecule (960 Da), so it will enter the cell even when little cell membrane damage occurs. Certain toxins are able to produce pores in the cell membrane through which TB enters (Tran et al. 2011). Although the cells are still viable, the impression is that the cells are non-viable. So the coloring may thus indicate little cell membrane damage while the cells are still viable. Also should be remarked that TB emits red fluorescence when binding with chitin and β-glucan present in yeast cell walls (Liesche et al. 2015).

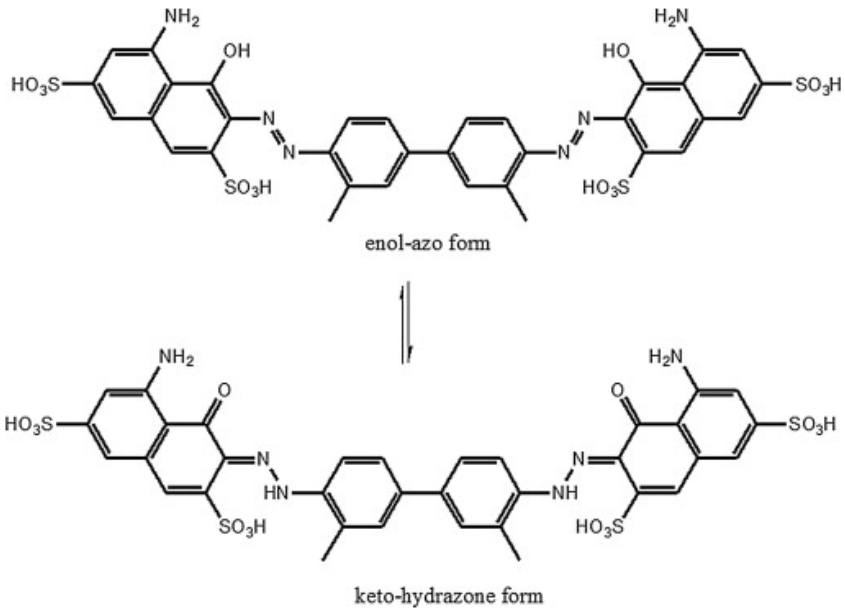

Figure 6: Structure of Trypan Blue in enol-azo and keto-hydrazone form. Source: Graham et al. (2013).

Rose Bengal

Rose Bengal and Phloxin B are two examples of xanthene dyes (Kawabata and Umemura 1997). Xanthene dyes exist in several halogenated derivatives (Figure 7 and 8). Rose Bengal is not an accurate coloring method for testing the viability of yeast cells. The dye colors viable cells, but also non-viable cells in some extent (Bernhard et al. 2006). Although the dye is still used often because it is cheap and easy-to-use (McCorkle et al. 1997).

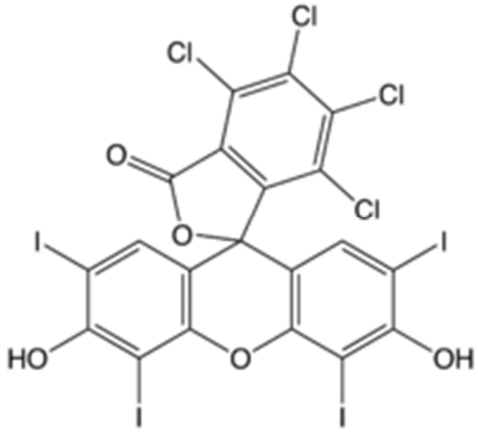

Figure 7: Structure of Rose Bengal: A xanthene dye. Adapted from Kawabata and Umemura (1997).

Phloxin B

Phloxin B is another xanthene dye to assess the viability of yeast cells. Both viable and non-viable cells are absorbing the red coloring agent. But only viable cells are able to pump Phloxin B back to the outer space (Minois et al. 2005). Therefore viable cells are colorless.

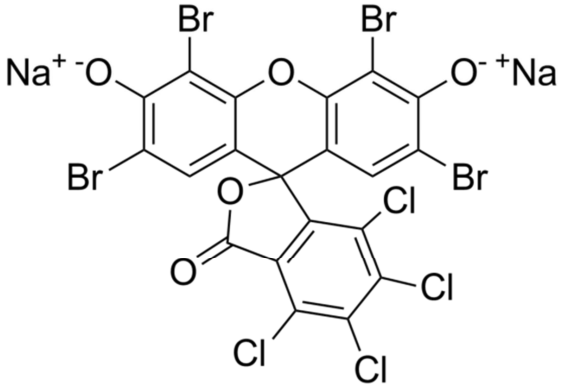

Figure 8: Structure of Phloxin B: A xanthene dye. Adapted from Kawabata and Umemura (1997).

1.4.2.2 Fluorescent stains

Fluorescent stains can be classified in several categories. The first category, stains are converted into a fluorescent product when entering metabolic active cells. In the second category the staining depends on the transmembrane electrochemical potential of the yeast cell which reflects the viability and the vitality in some extent (Heggart et al. 2000).

Mg-ANS

Mg-ANS enters the cell when damaged and binds upon cytoplasm proteins emitting a green fluorescence. It is the recommended method by *ASBC* (Middlekauff et al. 1981). Unwise the conventional MB staining with Mg-ANS is also accurate when measuring lower viabilities (Heggart et al. 2000).

Propidium iodide

A useful fluorescent dye is propidium iodide (Figure 9). Likewise Mg-ANS the viable cells are able to exclude propidium iodide. It enters the non-viable cells and when binding to the nucleic acids it emits red fluorescence (Davey and Hexley 2010).

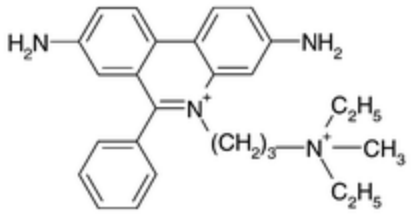

Figure 9: Structure of Propidium Iodide. Source: Kogi et al. (2002).

Fluorescein-diacetate (FDA)

FDA is able to penetrate the cell membranes of both viable and non-viable cells. Viable cells are able to hydrolyze FDA with esterases resulting in a green fluorescence (Heggart et al. 2000). Non-viable cells do not have esterase activity and therefore FDA has no fluorescence as result.

FUN-1

FUN-1 is able to penetrate the cell membrane of both viable and nonviable cells and provoke green fluorescence in the cytosol. In case of metabolic active cells, FUN-1 is metabolized by *S. cerevisiae* and is accumulated in the vacuole as a form of red-orange fluorescent cylindrical intravacuolar structures (CIVS) (Millard et al. 1997). So, nonviable cells emit bright green fluorescence and living cells vague green fluorescence with bright red-orange CIVS (Heggart et al. 2000).

1.4.2.3 Flow cytometry

Flow cytometry is used for detecting a large amount of stained cells one at a time. Mostly it is used to detect fluorescent cells. When a fluorescent cell passes through the laser beam it emits fluorescent side scatter (Figure 10). The non-fluorescent cells do not emit fluorescent side scatter. The side scatter is detected and converted into data with specialized software. Flow cytometry is able to determinate automatically and fast the amount of stained cells and thus also the amount of viable cells (Davey and Hexley 2010).

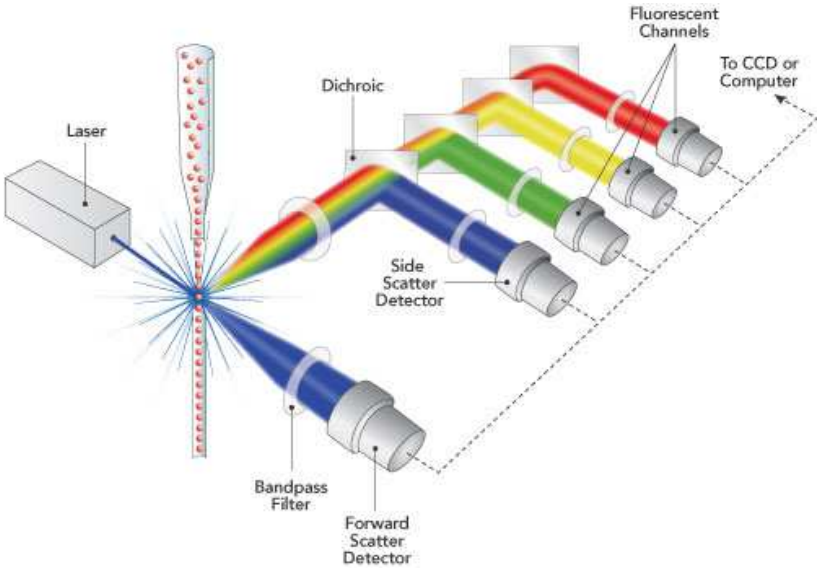

Figure 10: Flow cytometry as a detection method of stained cells.

2 Material and methods

In this section the material and methods are listed used in order to improve the yeast slide culture technique.

2.1 Strains, media and culture conditions

In this work, a laboratory (BY4741) and an industrial brewery (NCYC 1214) strain of *Saccharomyces cerevisiae* were used (Table 1). The strains were purchased from EUROSCARF collection (Frankfurt, Germany) and National Collection of Yeast Culture (United Kingdom), respectively.

Table 3: *S. cerevisiae* used in the present work

Reference	Strain	Collection
BY4741	Laboratory strain	EUROSCARF
NCYC 1214	Ale brewing flocculent strain	National Collection of Yeast Culture

Both strains were stored at 4 °C on YEPD agar slants (5 g/L yeast extract; 10 g/L peptone; 20 g/L glucose and 15 g/L agar). The yeast cells were grown in YEPD broth (10 g/L yeast extract; 20 g/L peptone and 20 g/L glucose).

For obtaining cultures in exponential phase, a pre-culture of 10 mL of YEDP broth in a 100 mL Erlenmeyer flask was inoculated with 1-2 loops of yeast and incubated for 8 h at 150 rpm and 25 °C. Cultures were obtained by inoculating 40 ml of YEPD broth in a 100 ml Erlenmeyer flask with the appropriate volume of pre-culture, in order to obtain an $OD_{(600nm)}$ ~ 1.0 after 15-16 h of incubation at 25 °C and 150 rpm.

2.2 Measuring of number of cells

The cell concentration was measured spectrophotometrically (Unicam, Helios Y) after appropriate dilution in water or 10 mmol/L EDTA (NCYC 1214) and using deionized water as blank.

For the strain BY4741, the cell concentration was determined using the following equation:

$$Number\ of\ cells * 10^6/ml\ = 20.24 \text{ x } \mathrm{OD}_{(600nm)} - 0.1348$$

For the strain NCYC 1214, the cell concentration was determined using the following equation:

$$Number\ of\ cells * 10^6/ml\ = 10.41 \text{ x } \mathrm{OD}_{(600nm)} - 0.060$$

2.3 Preparation of cell suspensions

After growth, cell suspensions were centrifuged (2000 x *g*, 5 min) and then washed twice with 20 mL deionized water or EDTA 30 mmol/L (strain NCYC 1214). Finally the cells were resuspended in 10 mL deionized water. All procedure took place under sterile conditions.

2.4 Stress conditions

Two stresses were applied. Yeast was stressed in ethanol or aged in water.

Ethanol

Cells were stressed in ethanol by adding the appropriate volume of cell suspension (final concentration of 1 x 10^7 cells/mL) in 10.0 mL phosphate buffer solution (PBS) 20 mM, pH 6.0. Then, ethanol (20% (v/v)) was added and the final volume of 20.0 mL was completed with water. Cells were incubated for 120 min, at 150 rpm and 25ºC. All procedure was done in sterile conditions.

Water

Cells were aged in water by adding the appropriate volume of cell suspension (final concentration of 1 x 10^7 cells/ml) to 7.5 ml PBS buffer 20 mM, pH 6.0. Then the final volume of 15.0 ml was completed with deionized water. Cells were incubated for 48 h, at 150 rpm and 25ºC. All procedure was done in sterile conditions.

After exposure to the stress conditions, cells were harvested by centrifugation (2000x *g*, 5 min) and washed with deionised water. Subsequently, the cells were resuspended in deionized water.

2.5 Slide culture technique

For preparing slide cultures, a defined volume of YEPD agar (12.5 or 25 mL) was poured into sterile 90-mm plastic Petri dishes and allowed to solidify. Two different compositions of YEPD agar were tested (Table 2). Then a layer of ~20x20 mm YEDP agar was used as growing substrate, inoculated with 20 µL of cell suspensions containing: $5x10^4$; $1x10^5$ or $2x10^5$ cells/mL; subsequently, cells were covered with a 24x24 mm coverslip and placed in a wetting chamber (100 µL water). All procedure was carried out in sterile conditions and the slides were incubated at 25 °C and observed under microscope after 6, 16, 20 and 24 h. The cells that gave rise to a micro-colony (4 cells or more) were deemed as viable. Single, double or triple cells were considered as non-viable. In each experiment and for each condition the counting was performed in duplicate (200 cells were scored in two culture medium blocks).

Table 4: Composition of YEPD agar for slide culture technique.

	Yeast extract	Peptone	Glucose	Agar
Recipe 1	5.0	10.0	20.0	15.0
Recipe 2	10.0	20.0	20.0	20.0

2.6 Budding index and short fermentation assay

A defined volume of cell suspension was added to 10.0 mL of double concentrated YEDP broth media. Then the final volume of 20.0 mL was completed with water in order to obtain a final cell concentration of $5x10^6$cells/mL. Cells were incubated at 25 °C for 6 h. The percentage of budding cells was determined microscopically at time 0 and 6 h. In each experiment, three samples of at least 300 cells (total $\geq$ 900 cells) were scored in randomly selected microscope fields. Additionally, the number of times of initial cell concentration after 6h was determined (short fermentation assay), using YEDP broth as blank.

The increase in cell concentration after 6 h was determined using the following equation:

$$Increase\ in\ cell\ concentration\ after\ 6h = \frac{C_{6h} - C_{0h}}{C_{0h}}$$

C_{0h} and C_{6h} correspond to the cell concentration at zero time and after 6h, respectively.

2.7 Effect of dyes on yeast cell growth

Inhibition effects of dyes on yeast growth were tested by monitoring cell concentration in different concentrations of each dye. First, a volume of 100 µL of yeast cells suspended in double concentrated YEPD broth media were placed in quintuplicate on a 96-well microplate (Orange Scientific) followed by 100 µL of double concentrated dye, performing a final volume of 200 µL with a cell concentration of 1 x 10^6 cells/mL. The microplates were incubated at 25ºC, in the dark. After 24h, the optical density was measured in a PerkinElmer (Victor3) microplate reader at a wavelength of 600 nm and corrected by subtracting the absorbance of culture medium with a given dye concentration.

For the yeast strain BY4741 the cell concentration was determined using the following equation:

$$Number\ of\ cells\ x\ 10^6/ml\ = 83.24\ \mathrm{X}\ \mathrm{OD}_{(600nm)} - 0.2599$$

Trypan blue

Yeast cells were incubated with TB solution (Aldrich), at the following concentrations: 0; 1.25x10^{-6}; 1.25x10^{-5}; 1.25x10^{-4}; 1.25x10^{-3}; 1.25x10^{-2}; 0.025; 0.05; 0.1; and 0.2 % (w/v).

Calcofluor white

Cells were incubated with Calcofluor white M2R (Sigma, Steinheim, Germany) with a final concentration of 0; 0.31; 0.63; 1.25; 2.5 and 5.0 µg/mL.

2.8 Microscopic observations of stained yeast cells

Cells were observed after 24 h of incubation. As positive control, dead cells were obtained by heating healthy cells at 65ºC for 1h. For both dyes, healthy cells (without any stress treatment) were also used as control.

Trypan blue

TB stained cells were observed with a Leica DLMB microscope by light and epifluorescence, equipped with a HBO-100 mercury lamp and a filter set I3 from Leica [excitation filter BP 450-490, dichromatic mirror 510 and suppression filter (long-pass filter, LP) LP 515].

Calcofluor white

Yeast cells were visualized by an epifluorescence microscope equipped with a filter set A (excitation filter BP 340-380, dichromatic mirror 400 and suppression filter LP 425) from Leica.

The images were acquired with a Leica DC 300F camera using N plan objectives and processed using Leica IM50 Image Manager software.

2.9 Calcofluor white as an aid for the slide culture technique

YEPD agar with CFW was prepared by adding CFW (final concentration of 2.5 µg/mL) immediately before pouring the 12.5 mL of medium into the Petri dish. The slides were prepared and observed with epifluorescence as previously described in section 5 and 7, respectively.

2.10 Reproducibility of the results and statistical analysis

The data are expressed as the means ± standard deviation (SD). Statistical differences were tested by one-way ANOVA followed by Tukey-Kramer multiple comparison method.

3 Results and discussion

Yeast slide culture technique has been used in the assessment of cell viability (Lentini 1993). However, for its large application, this technique should also be highly reproducible and easy-to-use. In this work, an effort was made to optimize and standardize all parameters of slide culture technique. Additionally, the use of dyes in combination with the yeast slide culture technique was evaluated for a better visualization of the cells. Finally the optimized yeast slide culture technique was applied to yeast cells in three different conditions (healthy, aged and ethanol stressed cells) and compared to other assessments for validating the improved technique.

3.1 Optimization of slide culture technique

This approach used regular slides with thin agar blocks as growing substrate and added cell suspension on top of it (Figure 11). Regular slides are cheaper than hemocytometer slides, being preferred. For avoiding an irregular surface that can impair cell counting, a defined volume of YEPD agar was poured in Petri dishes and, after solidification, 20x20 mm blocks were cut with a scalpel. YEPD agar blocks with a flat surface were obtained, facilitating the focus with the microscope. 20 µl of cell suspension were spread on the agar block, and then the slides were placed in a wetting chamber to prevent the drying of the growth media and incubated at 25 °C.

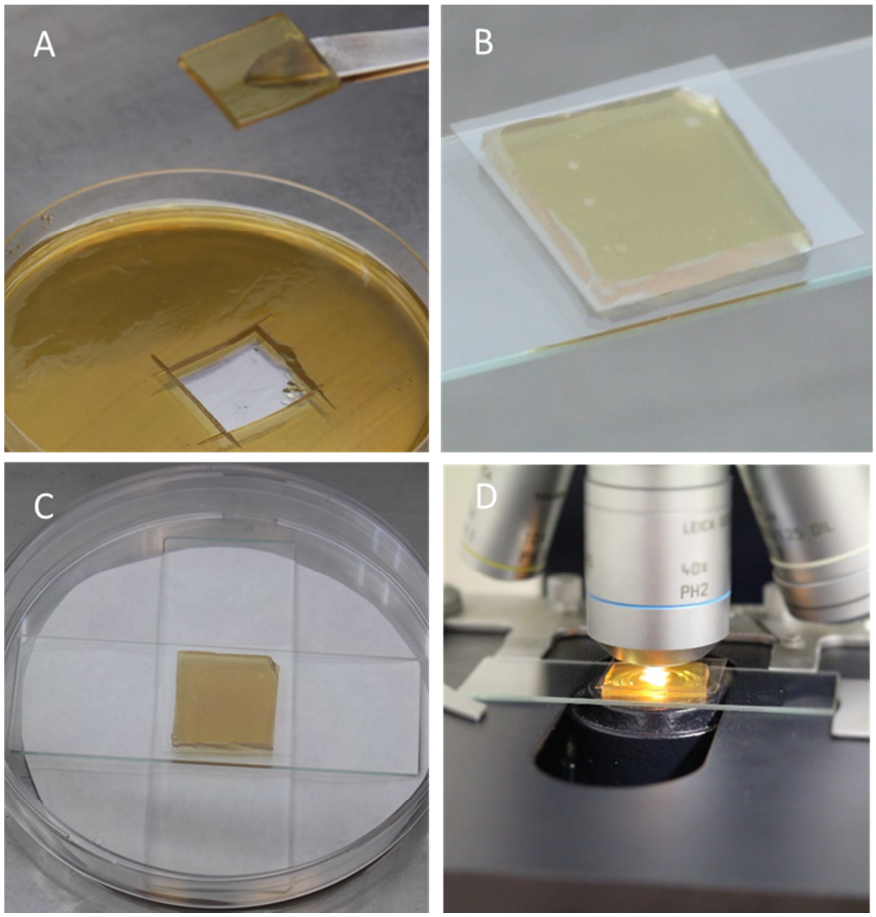

Figure 11: Slide culture technique for the assessment of *S. cerevisiae* viability: a general approach. (A) 20x20 mm YEPD agar blocks were cut with a scalpel and placed upon a regular slide. (B) 20 µl cell suspension was added on the YEPD agar block and the slide was covered with a coverslip. (C) The slide was placed in a wetting chamber and incubated at 25 °C. (D) Microcolonies were counted with the help of a microscope.

Preliminary experiments to set the working conditions were done with cells of the laboratory strain *S. cerevisiae* BY4741. The following parameters of the yeast slide culture technique were evaluated and improved: YEPD agar recipe, thickness of YEPD agar layer and humidity of wetting chamber.

An initial YEPD agar recipe of 5 g/L of yeast extract, 10 g/L of peptone, 20 g/L of glucose and 15 g/L of agar was used, but this medium did not present the desired strength. Additionally the yeast viability was underestimated. The cells were not properly fixed in the medium and the microcolonies tended to part from each other resulting in false nonviable cells on the observation with microscope. For overcoming these problems another recipe with more nutrients and agar content (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar) was adopted.

The thickness of the YEPD agar layer was defined having in account that the block could not be very thin for assuring nutrients availability and reduced dehydration. A volume of 25 mL of YEPD agar was poured in 90 mm diameter Petri dish and tested, but this layer presented some difficulties on focusing during cell counting. A thin block was performed with a volume of 12.5 mL of YEPD agar and eliminated this limitation.

For preventing dehydration, the block was placed in a wetting chamber with 100 µL of water. This allowed to maintain wet the medium even after 24 h of incubation. Higher volumes caused water condensation on the slides which was unwanted.

The slide incubation time, cell concentration and the counting criterion of the microcolonies are critical points of the assay. The incubation time should be as short as possible, but still enough for giving enough time to bud. The viability of healthy cells was determined after 6, 16, 20 and 24 h of incubation, at 25 °C. The incubation time was proposed after the cell concentration and the counting criterion were defined.

The appropriate cell concentration was established for two strains of *S. cerevisiae*: a laboratory (BY4741) and an industrial brewery (NCYC 1214) strain. The cell concentration should be as high as possible to facilitate the counting, but should not allow the overlapping of the microcolonies. For the strain BY4741 three cell concentrations and four incubation times were tested being the results presented in Table 5.

Table 5: Evaluation of different cell concentrations of healthy *S. cerevisiae* (BY4741) for the optimization of the slide culture technique.

Time of incubation (h)	$5x10^4$ cells/mL	$1x10^5$ cells/mL	$2x10^5$ cells/mL
6	+	+	+
16	+	+	+
20	+	+	±
24	+	±	-

The possibility to count was evaluated after 6, 16, 20 and 24 h of incubation: (+) micro colonies were easy to distinguish; (±) a small fraction of the micro colonies were covered partly, but counting was still possible; (-) counting was impossible because of overlapping.

A concentration of $2x10^5$ cells/mL had not allowed the counting after an incubation of 24 h since the microcolonies were completely overlapped. A concentration of $1x10^5$ cells/mL was preferred when compared with $5x10^4$ cells/mL since a higher number of cells had facilitated the counting and the

problems of overlapping at 24 h were negligible. When a concentration of $1x10^5$ cells/mL of the laboratory strain (BY4741) was used the microcolonies could be counted between 6 and 24 h.

Considering the NCYC 1214 strain of *S. cerevisiae*, two different cell concentrations and four incubation times were tested. The observations are presented in Table 6.

Table 6: Evaluation of different cell concentrations of healthy *S. cerevisiae* (NCYC 1214) for the optimization of the slide culture technique.

Time of incubation (h)	$5x10^4$ cells/mL	$1x10^5$ cells/mL
6	+	+
16	+	+
20	+	±
24	+	-

The possibility to count was evaluated after 6, 16, 20 and 24 h of incubation: (+) micro colonies were easy to distinguish; (±) a small fraction of the micro colonies were covered partly, but counting was still possible; (-) counting was impossible because of overlapping.

After 24 h of incubation a concentration of $1x10^5$ cells/mL caused overlapping and the counting of microcolonies was impossible. A cell concentration of $5x10^4$ cells/mL allowed an easy count of the industrial strain NCYC 1214 between 6 and 24 h. The most suitable cell concentration could thus slightly differ between strains depending on the doubling time and the cell size.

In previous works nonviable cells were reported as only single cells (Gilliland 1959; Jenkins et al. 2011), but these studies demonstrated that nonviable cells could also be observed as more than one single cell especially when the cells died during the budding process. This is particularly problematic for yeast cells in exponential phase of growth. When a budding cell dies before dissociation occured, the double cell is nonviable although it may seem that the cell is viable (Orlean 2012). Regarding this, as criterion, a cell was considered viable if it had the capacity to form a microcolony of 4 or more cells. This implied that an observation of double or triple cells scored as nonviable.

Comparing the viability of healthy cells of laboratory and industrial strains obtained by the optimized slide culture technique (Figure 12), it was possible to observe that, after 6h of incubation, the viability was around 90 %. For the BY4741 strain the viability increased significantly (P<0.05) after extending the incubation to 16, 20 and 24 h, reaching the expected value of almost 100 %. Also for the NCYC 1214 strain the means reached almost 100 % after 16, 20 and 24 h of incubation. For the BY4741 strain no significant (P<0.05) difference in cell viability was observed between 16, 20 and 24 h of incubation.

An incubation time of 6h had not been enough to form microcolonies but the viability of healthy cells of the strains BY4741 and NCYC 1214 could be determined after 16 to 24 h of incubation. The slide culture technique improved in this work allows assessing yeast viability in less than one day.

As cells in different physiological conditions (namely stressed cells or in stationary phase of growth) could need a long time to recover and to form microcolonies, a safety margin was taken and, an incubation time of 24 h was chosen as a preliminary set incubation time.

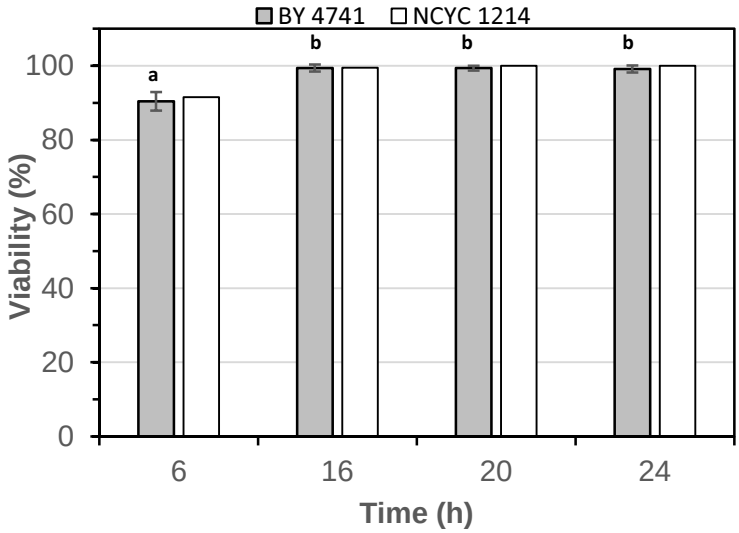

Figure 12: Viability of healthy cells of *S. cerevisiae* determined by the standardized slide culture technique. The viability (%) of the BY4741 strain was determined at least three times, in duplicate, using a concentration of $1x10^5$ cells/mL. The viability (%) of the NCYC 1214 strain was determined at least one time, in duplicate, using a concentration of $5x10^4$ cells/mL. The data are expressed as the means ± standard deviation (SD) for the BY4741 strain and as the means for NCYC 1214 strain. Statistical differences (for BY4741 strain) were subject to ANOVA followed by Tukey-Kramer multiple comparison method. The means with different letters were significantly different (P<0.05).

The incubation time and cell concentration could be adjusted for other strains and other physiological conditions.

3.2 Use of a stain to improve the slide culture technique

The possibility of combining the slide culture technique with a dye was investigated. Two dyes were evaluated: trypan blue and calcofluor white.

3.2.1 Trypan Blue

Trypan blue (TB) is a brightfield stain and has interesting extra properties to improve the slide culture technique. TB is able to penetrate cells with damaged cells membranes (Tran et al. 2011). When observed at microscope, nonviable cells are blue colored and viable cells are colorless. Combining TB with the slide culture technique, the viability assessment could be based on more than one definition of viability: 1) the ability to bud and 2) the integrity of the cell membranes. TB could also help on focusing on the yeast cells.

Additionally, when observed under fluorescence microscope TB also had the ability to emit red fluorescence. Liesche et al. (2015) had reported that TB can emit red fluorescence when binding with chitin and β-glucan present in yeast cell walls. Red fluorescence halos were observed and the bud scars were strongly stained indicating chitin accumulation. The dye could thus also help to visualize the yeast cell wall and scars. The fluorescent properties of TB were interesting as the fluorescent signal is very stable and it could improve focusing (Liesche et al. 2015).

Pierce (1962) already wanted to incorporate a staining method with the slide culture technique by adding methylene blue directly to the growth substrate of the slide culture. When the medium of the

slide culture is combined with a dye, first should be confirmed that the dye does not inhibit the cell growth. In this work, the yeast cell growth was monitored in YEPD broth with TB concentrations of $1.25x10^{-6}$; $1.25x10^{-5}$; $1.25x10^{-4}$; $1.25x10^{-3}$; $1.25x10^{-2}$; 0.025; 0.05; 0.1; and 0.2 % (w/v). Preliminary experiments indicated that growth inhibition was observed after 24 h with TB concentrations of $1.25x10^{-2}$ % (w/v) or higher. With TB concentrations below $1.25x10^{-2}$ % (w/v), it was very hard to distinguish between colored and not colored cells, with light microscope, after 24 h of incubation. More, TB tended to not only color nonviable but also viable cells in some extent after 24 h of incubation. The fluorescence signal with concentrations below $1.25x10^{-2}$ % (w/v) was also very weak.

As TB inhibited the growth with concentrations from $1.25x10^{-2}$ to 0.2 % (w/v) and concentrations from $1.25x10^{-6}$ to $1.25x10^{-2}$ % (w/v) were not sufficient to color the cells, the use of this dye was abandoned.

3.2.2 Calcofluor white

The fluorescent stain calcofluor white (CFW) can be an alternative to TB. CFW is not as stable as trypan blue (Liesche et al. 2015). Due to its instability CFW should be kept in the dark. This dye is not commonly used to assess viability, but rather to make the shape of yeast cells visible (Liesche et al. 2015). CFW emits blue fluorescence when interacting with the chitin in the yeast cell wall (Hoch et al. 2005). CFW could thus improve the yeast slide culture technique by facilitate the microcolonies focusing and was tested to combine with yeast slide culture technique.

Effect of calcofluor white on the growth of *S. cerevisiae*

Primarily, the effects of CFW on yeast cell growth were tested. It had to be confirmed that calcofluor white did not inhibit the growth for concentrations lower than 10 µg/mL (Boorsma et al. 2004). Yeast cell growth was monitored in YEPD broth with CFW concentrations of 0 – 0.31 – 0.63 – 1.25 – 2.5 – 5.0 µg/mL (Figure 13).

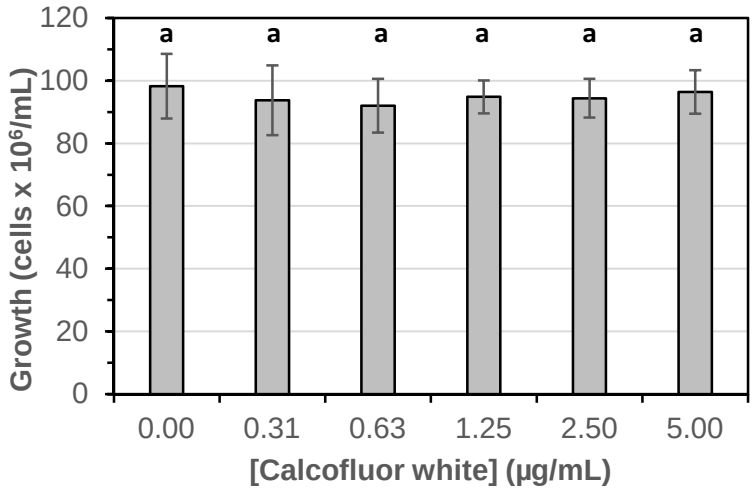

Figure 13: Effect of calcofluor white on the growth of *S. cerevisiae* (BY4741). Cells in YEPD broth ($1x10^6$ cells/mL) with 0 – 0.31 – 0.63 – 1.25 – 2.5 – 5.0 µg/mL of CFW were incubated at 25 °C. After 24 h of growth, the yeast cell concentration was determined. Three independent experiments were done in quintuplicate. The data are expressed as the means ± standard deviation (SD). Statistical differences were obtained by ANOVA followed by Tukey-Kramer multiple comparison method. The means with different letters were significantly different (P<0.05).

After 24 h of incubation at 25 °C, the final cell concentration of stained cells did not differ significantly *(P*<0.05) of unstained ones for all CFW concentrations tested. CFW did not inhibit cell growth with concentrations up to 5.0 μg/mL.

Effect of Calcofluor white on the cell structure of *S. cerevisiae*

CFW could have an effect on cell construction even when no growth inhibition occurred (Roncero and Durán 1985). Even when no growth inhibition was observed, cells could show aberrant alterations (Boorsma et al. 2004). So, the effect of CFW on normal cell morphology was evaluated.

Concentrations below 2.5 μl/mL of CFW resulted in partial coloring of the bud scars of the yeast cells. The coloring was not sufficient to facilitate the counting of the yeast slide culture technique although cell structure deformation was negligible. With a concentration of 2.5 μg/mL all the scars were colored and most of the cells had bright halos. Using 5.0 μg/mL of CFW, yeast cells (live and dead) were observed as bright blue halos. 2.5 μl/mL and 5.0 μl/mL CFW were suitable to color the cells and a slight difference could be observed between dead and living cells. When using 5 μg/mL CFW thick septa caused by chitin deposition and abnormal cell structures were observed. These results suggested that 2.5 μg/mL of CFW was a suitable concentration to color yeast cells after 24 h of incubation in YEPD broth medium, as no growth inhibition or structural alterations were observed.

3.3 Application of yeast slide culture technique on *S. cerevisiae* in different physiological states

The standardized slide culture technique (with and without CFW) was applied to *S. cerevisiae* BY4741, in three different physiological conditions: 1) healthy cells; 2) cells stressed in ethanol and 3) cells aged in water.

Despite, 24h had been set as the defined incubation time for healthy cells; an incubation time of 6 h was also tested for proving that the adding of CFW has no effect on viability assessment (Figure 14).

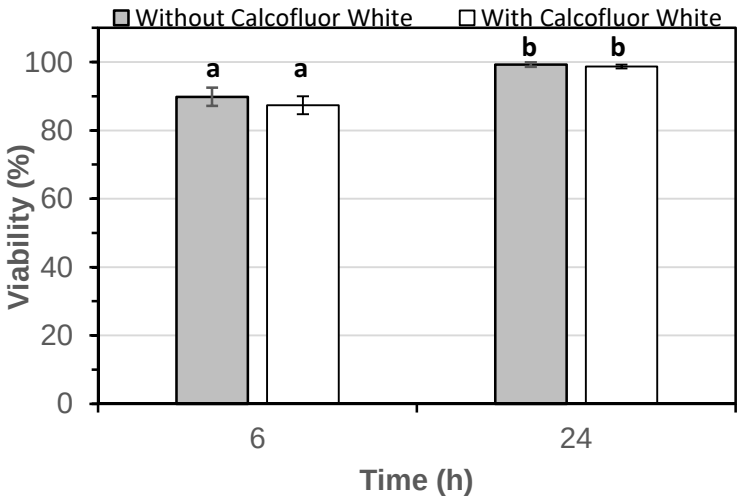

Figure 14: Viability of healthy cells of *S. cerevisiae* BY4741 determined by the standardized slide culture technique [with and without CFW (2.5 µg/mL)]. The slides with an initial yeast cell concentration of 1 x 10^5 cells/ mL were observed after 6 and 24 h of incubation at 25 °C. Three independent experiments were performed and counting in duplicate. The data are expressed as the means ± standard deviation (SD). Statistical differences were subject to ANOVA followed by Tukey-Kramer multiple comparison method. The means with different letters were significantly different (*P*<0.05).

The viability of healthy cells by the slide culture technique after 6 h of incubation (89.8 %) was not significantly different (*P*<0.05) from the healthy cells stained with CFW (87.3 %). The same occurred for 24 h of healthy cells; CFW (2.5 µg/mL) did not significantly (*P*<0.05) influence the viability and facilitated focusing when counting the microcolonies. Cell scars were stained and the cell walls became visible as bright blue halos (Figure 15). The observed cells had normal cell structures. CFW (2.5 µg/mL) could thus be used in combination with the yeast slide culture technique when assessing healthy cells after an incubation of 24 h.

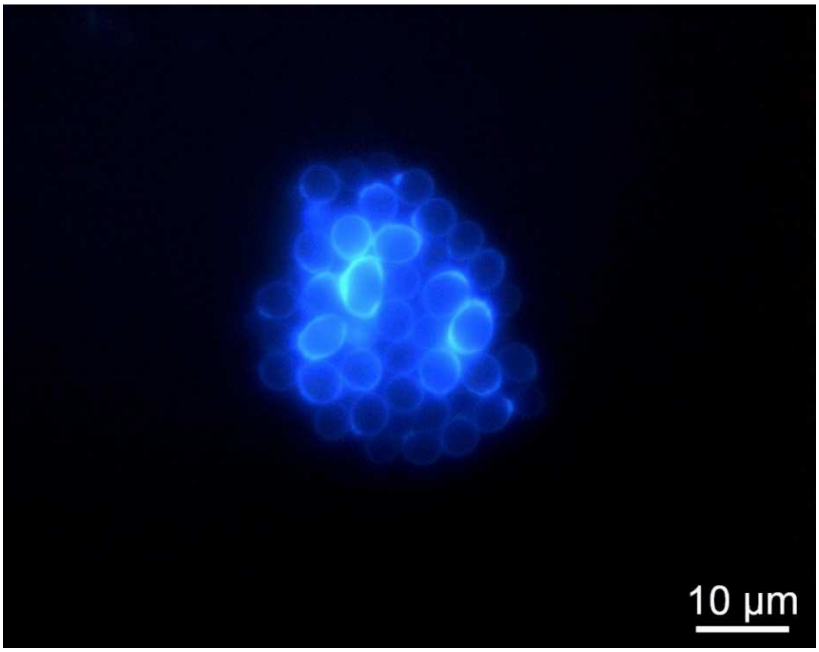

Figure 15: Microcolony morphology of *S. cerevisiae* BY4741 obtained by slide culture technique stained with CFW. Viable yeast cell after 24 h of incubation gave rise to a micro colony. Adding 2.5 µl/mL CFW to the growth media of the slide culture resulted in bright blue stained cells. Scars and cell wall are colored more intense as CFW reacts with the chitin.

3.3.1 Slide culture applied to ethanol stressed yeast cells

The combination of the slide culture technique with CFW was standardized for healthy cells of *S. cerevisiae* (BY4741). The influence of stress on yeast cells and their implication on the slide culture technique had to be investigated. Ethanol stress is very common for *S. cerevisiae* in alcoholic beverages and bio-ethanol production (Ben Chaabane et al. 2006; Lodolo et al. 2008). The effect of 20 % (v/v) ethanol on cell viability was evaluated (Figure 16) by slide culture technique with and without CFW after an incubation time of 6 and 24 h.

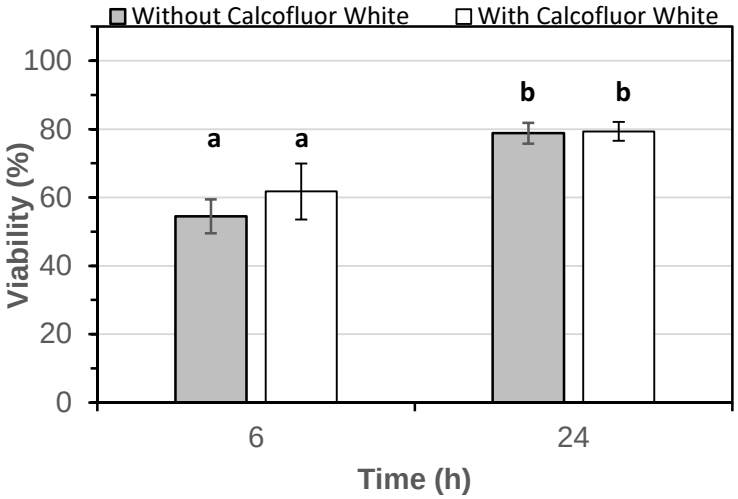

Figure 16: Viability of *S. cerevisiae* BY4741 exposed to 20 % (v/v) ethanol, determined by slide culture technique [with or without the addition of 2.5 µg/ml CFW]. Cells were stressed in 20 % (v/v) ethanol for 2 h, at 25ºC. The slides were observed after 6 and 24 h of incubation, at 25 °C. Three independent experiments were performed and counting in duplicate. The data are expressed as the means ± standard deviation (SD). Statistical differences were subject to ANOVA followed by Tukey-Kramer multiple comparison method. The means with different letters were significantly different (P<0.05).

The cell viability after an incubation of 6 h was not significantly (P<0.05) different from the cells not stained (54.5 %) or stained with CFW (61.8 %). When cells were incubated for 24 h with or without CFW, no viability changes were also observed. For ethanol stressed cells, 2.5 µg/mL CFW did not significantly (P<0.05) influence the viability and facilitated focusing when counting the microcolonies. Stressed cells that remained viable had a similar stain as healthy cells. Nonviable cells that did not form a microcolony were stained more diffuse as observed in preliminary experiments. Fluorescent background should be avoided in order to properly visualize the nonviable cells. CFW (2.5 µg/mL) could thus be used in combination with the yeast slide culture technique when assessing cells stressed in ethanol.

The applying of slide culture technique to ethanol stressed cells was confirmed that 6 h of incubation was not sufficient for determine yeast viability. The viability after 6 h of incubation was significantly (P<0.05) lower than after 24 h and was thus seriously underestimated. Further work should be done in order to decide if 24 h is an appropriate incubation time. Short incubation times of 16 and 20 h should also be tested as well as times higher than 24 h could be considered.

3.3.2 Slide culture applied to cells aged in water

Starvation is a common stress imposed to yeast cells in fermentative industries. Thus, the effect of aging the cells in water on yeast viability and the effect of CFW was evaluated (Figure 17).

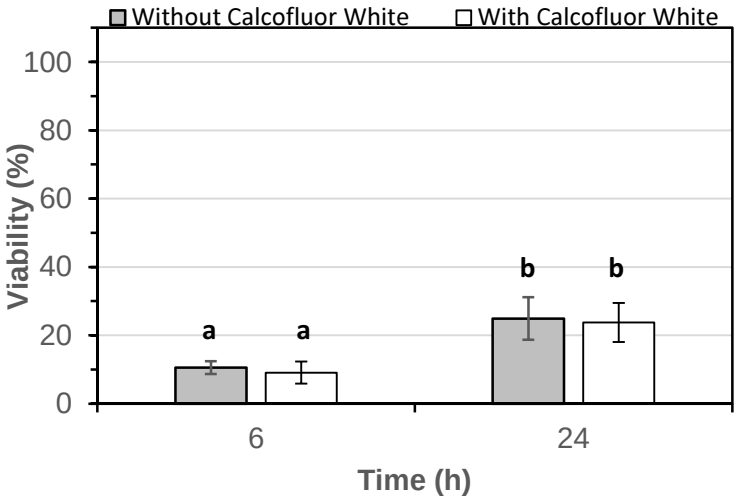

Figure 17: Viability of *S. cerevisiae* BY4741 cells aged in water, determined by the slide culture technique. Cells were aged for 48 h in water, at 25ºC. The slides were observed after 6 and 24 h of incubation at 25 °C. Three independent experiments were performed and counting in duplicate. The data are expressed as the means ± standard deviation (SD). Statistical differences were subject to ANOVA followed by Tukey-Kramer multiple comparison method. The means with different letters were significantly different (P<0.05).

The cell viability did not differ significantly (P<0.05) when using the CFW stain. An incubation time of 6 h seriously underestimated (10.6 % and 9.1 % without or with CFW, respectively) the yeast cell viability. The viability at 24 h was significantly (P<0.05) higher (24.9% without and 23.8 % with CFW). Also here further work should be done in order to decide if 24 h is an appropriated incubation time. The use of CFW (2.5 µg/mL) was justified when assessing aged cells with the yeast slide culture technique.

3.4 Comparison of slide culture technique with short fermentation test and budding index determination

Standardized slide culture technique was compared to two other assessments commonly used in the industry: the short fermentation test and the budding index. The techniques are often used in industries because are very easy to perform.

3.4.1 Short fermentation test

The short fermentation test is a common vitality assessment in breweries and was operated by a small scale fermentation in a short time (6 h). The increase in cell concentration was compared to the initial cell concentration (Figure 18). The short fermentation test evaluates the capacity of the yeast cells to reproduce.

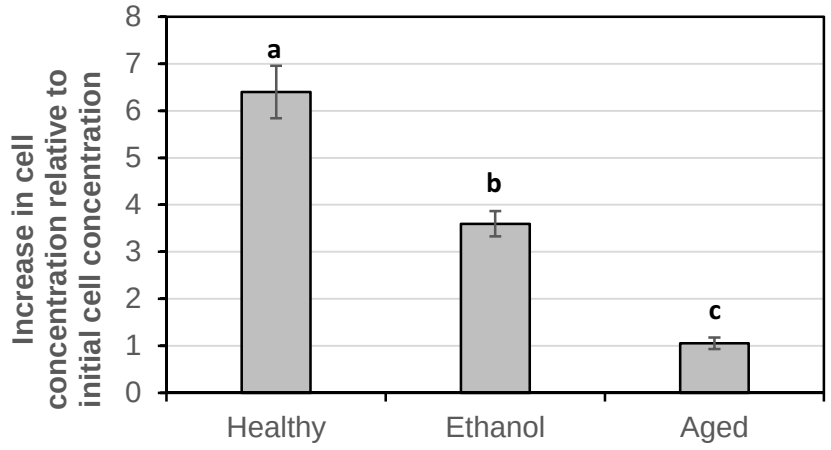

Figure 18: The short fermentation test of *S. cerevisiae* BY4741 in three different physiological conditions. 20 mL of YEPD broth with a final yeast concentration of ~5x10^6 was incubated for 6 h at 25 °C. The increase in cell concentration relative to the initial cell concentration was determined. Three independent experiments were performed and cell concentrations were measured in quadruplicate. The data are expressed as the means ± standard deviation (SD). Statistical differences were subject to ANOVA followed by Tukey-Kramer multiple comparison method. The means with different letters were significantly different (*P*<0.05).

The increase in cell concentration relative to the initial cell concentration was 6.4 for healthy cells. This was significantly (*P*<0.05) higher than cells subjected to stress. The values also differ significantly (*P*<0.05) for cells stressed in ethanol (3.6 times) and cells aged in water (1.1 times). The tendency obtained in the short fermentation assay was similar to obtained with the standardized yeast slide culture technique. The short fermentation test could give a rough estimation of the viability.

3.4.2 Budding index

The budding index is common used to assess viability of yeast in breweries. The viability is estimated by counting the percentage of budding cells (Figure 19).

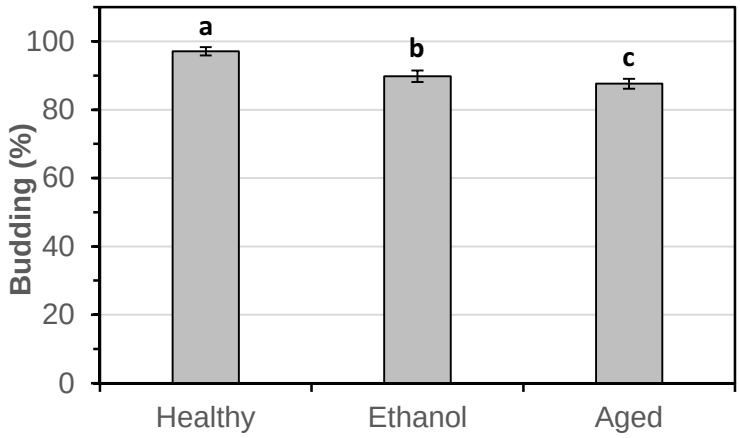

Figure 19: The budding index of cells of *S. cerevisiae* BY4741 in three different physiological conditions. 20 mL YEPD broth with a final yeast concentration of approximately 5x10^6 was incubated for 6 h at 25 °C. Three independent experiments were done counting each time more than 300 cells. The data are expressed as the means ± standard deviation (SD). Statistical differences were subject to ANOVA followed by Tukey-Kramer multiple comparison method. The means with different letters were significantly different (*P*<0.05).

After 6 h of incubation the budding index was significantly (P<0.05) higher for healthy cells (97.1 %) than for cells exposed to stressed conditions. After 6 h, the budding index of cells stressed in ethanol (89.8 %) was significantly (P<0.05) higher than of cells aged in water (87.6 %).

The budding index, as the slide culture technique, is based on the ability of the cell to form buds. The budding assay had shortcomings determining the yeast viability. The results obtained with the budding index strongly depended on the yeast conditions before the stress was imposed. It should be noted that not all observed budded cells are viable for the same reasons stated in section 2.2.1. Despite the ease of the budding assay it could only be used to give a rough estimation of the viability. After 6 h the same tendency in viability was reflected as obtained with the yeast slide culture technique. But the difference in viability was less clear with the budding assay.

In further studies the standardized yeast slide culture technique could also be compared with the standard plate count method. The standard plate count method is based on the same principles as the yeast slide culture technique (Pierce 1962). Still the comparison could be useful because the standard plate count was a common practice in laboratories.

4 Conclusions

In this work, an improved slide culture technique was developed. A thin 20x20 mm agar block was used as growing substrate and 20 µL of cell suspension was added on its top. Incubation was performed in a wetting chamber, at 25 °C, up to 24 h. The addition of 2.5 µg/mL of calcofluor white (CFW) to YEPD medium benefited the microcolony counting in the slide culture technique.

The viability of yeast in the three different physiological conditions (healthy, ethanol stressed and aged cells) was assessed by slide culture technique with and without CFW. No significant difference was observed when applying CFW to the yeast slide culture technique. Comparing yeast viability obtained by the developed technique with conventional techniques, namely short fermentation and budding index, similar results were obtained.

To conclude, slide culture technique combined with CFW seems to be a promissory alternative to conventional methods for the evaluation of yeast cell viability.

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